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Escherichia coli with Two Linear Chromosomes

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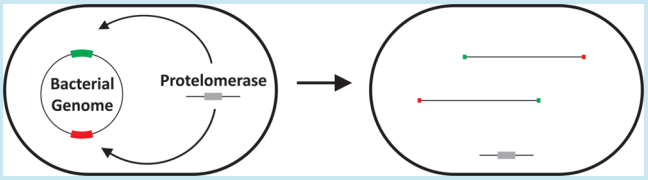
Supporting Information

ABSTRACT: A number of attempts have been made to simplify the synthesis of whole chromosomes to generate artificial microorganisms. However, the sheer size of the average bacterial genome makes the task virtually impracticable. A major limitation is the maximum assembly DNA size imposed by the current available technologies. We propose to fragment the bacterial chromosome into autonomous replicating units so that (i) each episome becomes small enough to be assembled in its entirety within an assembly host and (ii) the complete episome set should be able to generate a viable cell. In this work, we used the *telN*/*tos* system of bacteriophage N1 to show that the circular genome of *Escherichia coli* can be split into two linear chromosomes that complement each other to produce viable cells.



KEYWORDS: *genome engineering, chromosome linearization, genome assembly, chromosome manipulation*

Most of the industrial synthetic biology initiatives use *Escherichia coli* or other related bacteria including prokaryotic algae as production hosts. A few examples are Genomatica's 1,4-butanediol, Amyris' artemisinin, and biofuels made by BP, Exxon, and Dupont.[1,2] These and other commercial projects require extensive genome remodeling, which involves the generation and transfer of very large genetic elements including chromosomes. Proof of principle of some of these procedures has been achieved only in the context of relatively small chromosomes such as those of *Mycoplasma* species (0.6−1.08 Mbp) and *Prochlorococcus marinus* (1.66 Mbp), which were assembled in the yeast *Saccharomyces cerevisiae*.[3,4] Analyses of various large episomes, including concatemers of the above, suggested that yeast can stably maintain foreign DNA molecules of up to 2 Mbp.[5] Other highly recombinogenic and competent organisms, such as *Bacillus subtilis*, showed proficiency in chromosome assembly but were not capable of maintaining large autonomous replicating molecules.[6] Overall, no biological platform can both assemble and sustain the replication of episomes larger than 2 Mbp, a size that is significantly smaller than the chromosomes of most bacterial species including *E. coli*, most Gram (+) bacteria, and prokaryotic algae.

One way to overcome the limitation above is to split the prokaryotic chromosomes into autonomous replicating units small enough to be assembled in yeast and, at the same time, able to complement each other, thereby generating viable cells. To obtain an initial proof of concept for the workflow proposed above, we set out to investigate whether *E. coli*'s circular chromosome could be split into 2 smaller units without affecting cell viability.

***In Vivo* Approaches for Chromosome Fragmentation.** We could conceive two different approaches, at least, to split a circular chromosome *in vivo* into two or more episomes. First, unidirectional site-specific recombination may be applied between two or more DNA recognition sequences placed at strategic locations in the chromosome (Figure 1A). This task can be performed by transient induction of a variety of phage recombinases widely used in genome engineering.[7] This approach has major drawbacks. Primarily, recombination sequences must be added in pairs rather than individually. Second, to avoid unwanted rearrangements, the pairs must be perfectly orthogonal, which is not trivial to achieve. Third, the existence of pseudo recognition sequences in the genome together with suboptimal recombinase expression levels may corrupt the entire approach. Finally, the recombinase should be eventually eliminated to avoid potential further rearrangements.

An alternative approach would be to split the chromosome by means of the introduction of multiple linearization sites (Figure 1B). Proof of concept for *E. coli* linearization at a single site has been demonstrated by using two components of the bacteriophage N15: the telomerase occupancy site element (*tos*) and the protelomerase protein (TelN).[8] Briefly, the *tos* element is recognized and cut at staggered positions at an internal palindromic region by the TelN protein. Next, the single-stranded DNA regions are self-annealed, and, finally, the nicks are sealed by the TelN protein, producing two termini with hairpin structures (inset in Figure 1B). The linearization strategy has a few advantages over the site-specific recombination approach. First, *tos* elements could be added individually (rather than in pairs) on a sequential basis to obtain increasing number of linear subgenomes. Second, sequence orthogonality is not a prerequisite because identical *tos* elements may be repeatedly placed at different chromosomal locations. Third, extended *telN* expression does not generate genome rearrangements other than those specifically intended. Finally, it has been demonstrated that *E. coli* cells with a circular chromosome

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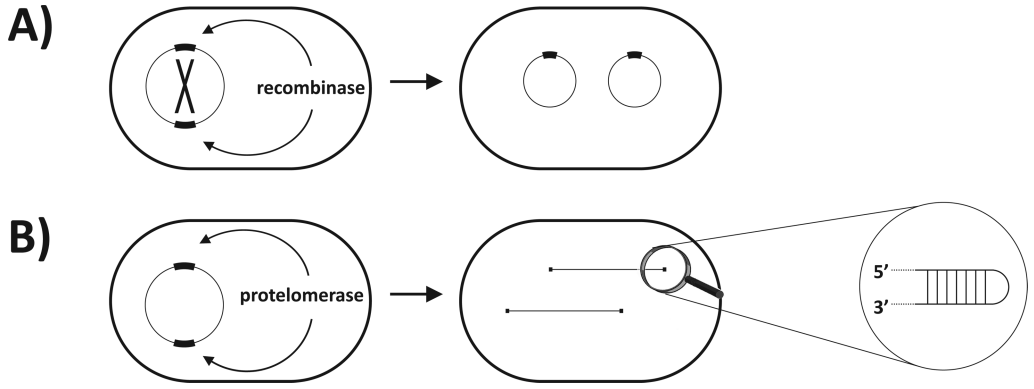

Figure 1. Chromosome fragmentation strategies. (A) Fragmentation using recombination sequences. A pair of compatible recombination sequences is inserted at appropriate sites in the chromosome (black boxes). Induction of a site-specific recombinase triggers recombination between the sites, generating two smaller circular episomes. (B) Fragmentation using *tos* elements and a protelomerase. Strategically located *tos* elements (black boxes) are cut and sealed upon protelomerase expression. The inset shows the topology of the ends of the DNA molecules after being processed by the protelomerase.

harboring two active replication origins grow with relatively normal growth rates and cell cycle parameters.[9] This information is particularly relevant because no matter which of the strategies above is followed, such a stage is an obligated step toward the final strain with multiple chromosomes.

RESULTS AND DISCUSSION

Generation of Strains Harboring Different Combinations of Genome-Engineering Elements. To identify the genetic requirements for the fragmentation of the *E. coli* chromosome, we generated a variety of strains with different replication and DNA processing elements inserted at different genome locations. First, we varied the number and locations of the *E. coli* origin of replication (OriC) and the N15 bacteriophage *tos* element. The wild-type *E. coli* OriC [OriC(wt)] is a 232 bp sequence located at the 84.5 min of the chromosome of the MG1655 strain. We inserted this sequence at several locations of the chromosome, naming those OriC1, OriC2, OriC3, and OriC4 (Figure 2A.). The N15 bacteriophage *tos* element is contained within a 564 bp DNA sequence harboring a series of inverted repeats centered on a large palindromic sequence.[10] Depending on the chosen genomic position, we named these elements *tos1*, *tos3*, *tos4*, *tos7*, and *tos8* (Figure 2A and Supporting Information). All of the strains (Figure 2B, strains 1−14) were viable even when some of them harbored two replication origins (Figure 2B, strains 2, 4, 6, 8, 10, 12, and 14). These additional replication origins were confirmed functional because they were able to sustain chromosome replication in the absence of OriC(wt) (Figure 2B, strains 3, 5, 7, 9, 11, and 13).

Strains were later transformed with the plasmid pJAZZ-OC, which expresses the bacteriophage N15 *telN* gene,[11] and selected on chloramphenicol (cam) LB plates. We expected that the resulting strains should have one or two linear chromosomes depending on the number of *tos* elements inserted in their genomes. Only those cells harboring one *tos* element in their genomes resulted in viable cells after transformation (Figure 2B, strains 1 and 14). These results suggest that at least one of the smaller chromosomes in those strains harboring two *tos* sites is either unable to replicate properly or partition accurately into the two daughter cells. Insufficient replication and partitioning factors might account for these results in an apparent analogy to plasmids belonging to the same incompatibility group.

Use of a Heterologous Origin of Replication. Consequently, we shifted our focus to origins of replication present in bacteria harboring multiple chromosomes. In almost all of the sequenced multipartite genomes, the different chromosomes appear to contain different origins of replication, which may prevent incompatibility between coresident chromosomes.[12] We then imagined that an *E. coli* cell with two essential chromosomes with different replication and partitioning requirements may be viable. For the proof of concept of the hypothesis above, we chose the *Vibrio cholerae* multi chromosome system, which has one primary (2.96 Mbp) and one secondary chromosome (1.07 Mbp).[13] Whereas the features of the replication origin of the primary chromosome are essentially the same as those of the *E. coli* chromosome, replication of the secondary chromosome is controlled by its own initiator RctB.[14] The genetic locus involved in replication includes the *rctB* gene and an array of repeats, which is reminiscent of that of some *E. coli* plasmids.[15] We then PCR amplified and cloned a 5.6 kb region encompassing the origin of replication of *V. cholerae*'s secondary chromosome[16] (for details, see the Methods and Supporting Information). We inserted this sequence into four different locations of the *E. coli* chromosome, naming those OriV1, OriV2, OriV3, and OriV4, and combined them with *tos* elements placed at various sites (Figure 2B, strains 15−22). In a few cases, the wild-type OriC was subsequently knocked out, indicating that OriV can sustain replication of the whole *E. coli* circular chromosome on its own (Figure 2B, strains 16 and 21). Upon transformation of the strains with two *tos* sequences inserted into their genomes with the plasmid pJAZZ-OC, only strains 20 and 22 yielded colonies on LB chloramphenicol plates (Figure 2B, strains 17−22). Out of these two, only strain 22 yielded a consistent number of colonies when repeatedly transformed with pJAZZ-OC. The chromosome sizes and cleavage sites of strain 22 could be unequivocally confirmed by PCR, southern blot, and pulse-field gel electrophoresis (Figure 2C−E). The physical characterization of the genome of strain 22 is consistent with a model where the original 4.6 Mbp circular *E. coli* chromosome is split into two linear chromosomes of 3.27 and 1.37 Mbp (Figure 2F).

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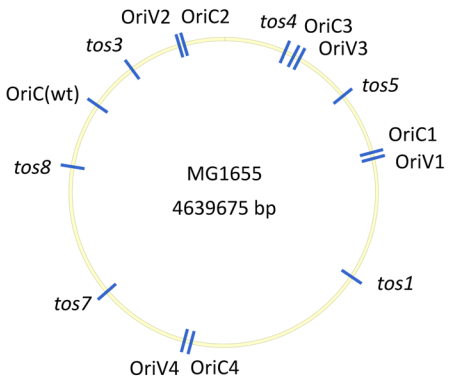
A)

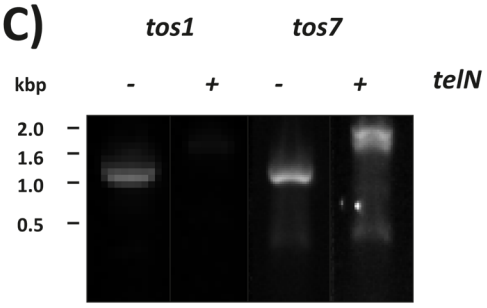


B)

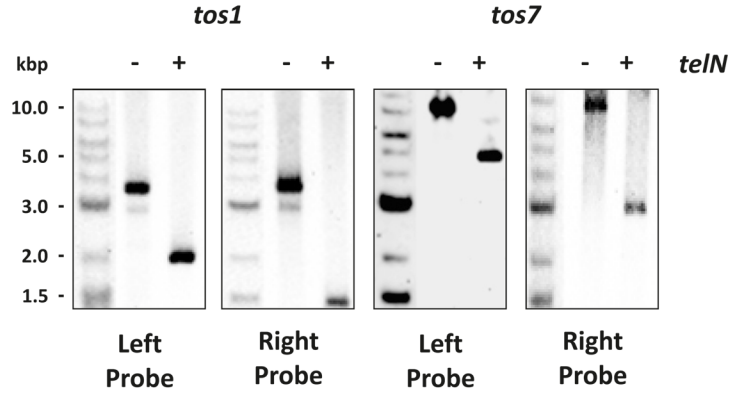
Strain number	Strain's relevant genotype	Strain viability		Anticipated chromosome sizes (Mbp)*
		without *telN*	with *telN*	
1	OriC(wt), *tos1*	Yes	Yes	4.64
2	OriC, OriC3, *tos1*, *tos3*	Yes	No	2.58 + 2.06
3	ΔOriC(wt), OriC3, *tos1*, *tos3*	Yes	N/A	N/A
4	OriC(wt), OriC3, *tos1*, *tos4*	Yes	No	1.29 + 3.35
5	ΔOriC(wt), OriC3, *tos1*, *tos4*	Yes	N/A	N/A
6	OriC(wt), OriC1, *tos1*, *tos3*	Yes	No	2.58 + 2.06
7	ΔOriC(wt), OriC1, *tos1*, *tos3*	Yes	N/A	N/A
8	OriC(wt), OriC1, *tos1*, *tos4*	Yes	No	1.29 + 3.35
9	ΔOriC(wt), OriC1, *tos1*, *tos4*	Yes	N/A	N/A
10	OriC(wt), OriC4, *tos1*, *tos3*	Yes	No	2.58 + 2.06
11	ΔOriC(wt), OriC4, *tos1*, *tos3*	Yes	N/A	N/A
12	OriC(wt), OriC4, *tos1*, *tos4*	Yes	No	1.29 + 3.35
13	ΔOriC(wt), OriC4, *tos1*, *tos4*	Yes	N/A	N/A
14	OriC(wt), OriC2, *tos1*	Yes	Yes	4.64
15	OriC(wt), OriV2, *tos1*	Yes	Yes	4.64
16	ΔOriC(wt), OriV2, *tos1*	Yes	No	(4.64)
17	OriC(wt), OriV3, *tos1*, *tos3*	Yes	No	2.58 + (2.06)
18	OriC(wt), OriV1, *tos1*, *tos3*	Yes	No	2.58 + (2.06)
19	OriC(wt), OriV4, *tos1*, *tos8*	Yes	No	2.62 + (2.02)
20	OriC(wt), OriV1, *tos1*, *tos5*	Yes	Yes (few colonies)	3.71 +(0.93)
21	ΔOriC(wt), OriV1, *tos1*, *tos5*	Yes	N/A	N/A
22	OriC(wt), OriV4, *tos1*, *tos7*	Yes	Yes	3.27 + (1.37)

*The size of the chromosome, whose replication is driven by the OriV element is indicated between parentheses

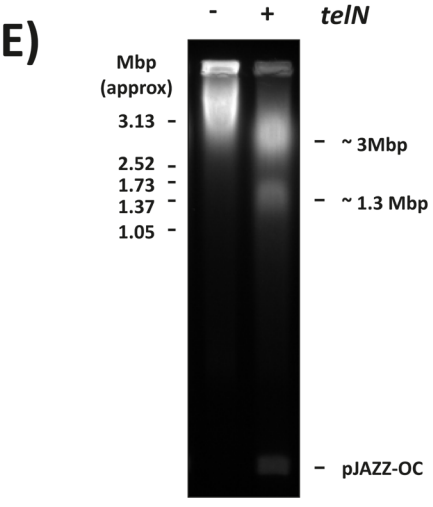
C)



D)



E)



F)

Figure 2. Genetic elements and chromosome topology of the strains generated in this work. (A) Relative location of the different genetic elements inserted into the chromosome of the *E. coli* strain MG1655. Elements were inserted into the genomes of different strains according to panel B. B) Relevant genotype, viability, and anticipated chromosome size of the strains generated in this work. The *telN* gene was introduced into the corresponding cells as part of the plasmid pJAZZ-OC. (C) PCR assay across the corresponding *tos* elements in strain 22 in the presence or absence of the *telN* gene. (D) Southern blot using digested genomic DNA extracted from strain 22 in the presence or absence of the *telN* gene. (E) Pulse-field gel electrophoresis using intact genomic DNA extracted from strain 22 in the presence or absence of the *telN* gene. (F) Anticipated topological configuration of the two chromosomes of strain 22 expressing the *telN* gene. Numbers represent the position in the corresponding chromosome. For additional details such as DNA sequences, precise location of the elements, probe composition, expected DNA fragment sizes, and protocols employed, see the Methods and Supporting Information.

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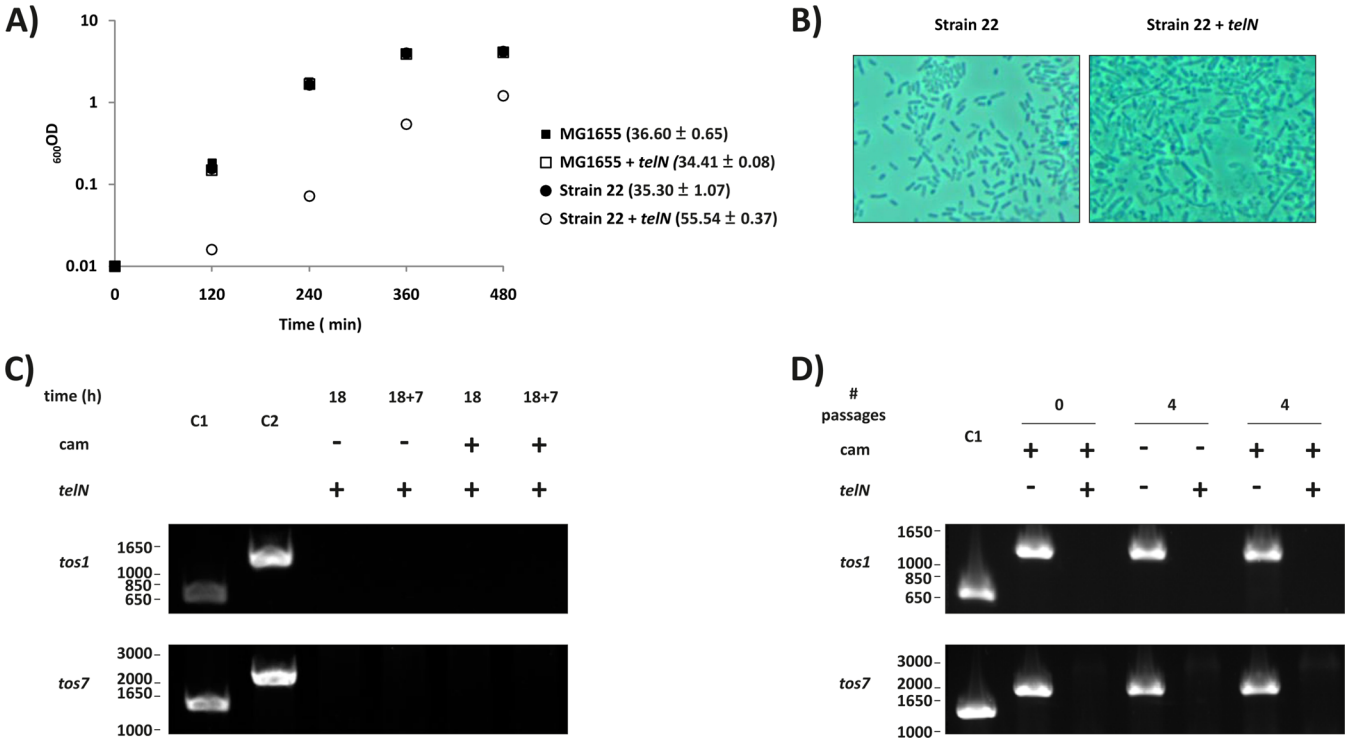

Figure 3. Characterization of strain 22 + *telN*. (A) Growth curves. Growth curves and doubling times were computed as indicated in the Methods. Numbers in brackets are doubling times, expressed in min, of the corresponding strains. In all cases, experiments were performed in duplicate with errors <3%. (B) Cell morphology. Overnight cultures from panel A were processed and visualized as described in the Methods. (C) Genome stability during culture. Aliquots of the cultures in panel A were subjected to PCR to verify chromosome fragmentation across the *tos1* and *tos7* DNA elements. Pairs of oligonucleotides flanking the corresponding *tos* sequences were used (for details, see the Supporting Information). Aliquots from the overnight seed culture (18 h) and from the last data point of the growth curve (18 h*) were used. Controls were overnight cultures of MG1655 and strain 22 (C1 and C2, respectively). (D) Long-term genome stability. Strain 22, either harboring or not harboring the *telN* gene, was restreaked the indicated number of times onto LB agar plates. Where indicated, the medium was supplemented with 10 μg/mL of chloramphenicol (cam). Isolated colonies were subjected to colony PCR using the oligonucleotides described in panel C.

Lytic and lysogenic replication modes for the bacteriophage N15 have been proposed.[17,18] The lysogenic mode is contemplated by two models: in one of them, the protelomerase processes the ends before completion of replication (mode 1), whereas in the second, replication is completed before the molecule is processed, generating a head-to-head dimer (mode 2). The lytic replication has been proposed to follow a rolling-circle strategy originating from the head-to-head dimer described above, and the concatemers are cleaved at the cos sites (mode 3). The replication of the chromosomes in strain 22 probably follows mode 1 because we were unable to detect the head-to-head dimer intermediate required for modes 2 and 3 (Figure 2D).

Phenotypes of Strain 22 Harboring a *telN* Gene. The aspect of the colonies of strain 22 + *telN* was indistinguishable from those of strain MG1655 and strain 22 (not shown). However, the growth rate of strain 22 + *telN* was approximately 1.6-fold slower than the other strains (Figure 3A). In addition, overnight cultures of strain 22 + *telN* contained approximately 10% of elongated cells (Figure 3B), a phenotype consistently observed regardless of the number of passages. These observations suggest that the cells with two linear chromosomes exhibit a slight cell division defect. Fluorescent microscopy studies will be required to determine whether this minor deficiency is related to chromosome duplication, partitioning, or cell segmentation. The slower growth rate of these cells would potentially offer an evolutionary advantage for faster-growing suppressors, which might have escaped genome fragmentation, by regenerating a single linear or circular 4.6 Mbp chromosome. To test this hypothesis, PCR amplification assays across both *tos* elements were performed. Whereas strain 22 exhibited the PCR fragment that indicates integrity of the *tos* region in all cases, we failed to detect the corresponding band when strain 22 + *telN* was used, even after 100 generations or four successive restreaks on LB agar (Figure 3C,D). The results remained the same when selective pressure against the source of *telN* (10 μg/mL cam) was lifted (Figure 3A,D). Overall, despite the slow grow rate, the strain with two linear chromosomes did not show any sign of genetic instability under our experimental conditions.

Overall, the results indicate that an *E. coli* strain harboring two linear chromosomes can be constructed and stably maintained under standard laboratory conditions. Although the data supports the concept we originally intended to prove, a fundamental question still remains open: what are the basic rules that allow viable chromosome fragmentation? Molecule size and nature and positioning of the replication and linearization elements are relevant, but additional determinants may be equally important. For instance, OriV, which in its natural host drives the replication and partitioning of a 1.07 Mbp circular chromosome,[16] can function in the context of the entire 4.6 Mbp circular *E. coli* genome, but it is apparently unable to sustain the replication and/or partitioning of a seemingly identical chromosome that adopts a linear topology (Figure 2B, strain 16). At the same time, none of the tested permutations that included OriV as the sole replication element

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present in 2 Mbp linear chromosomes resulted in viable strains (Figure 2B, strains 17−19). However, a combination designed to harbor a linear OriV-replicating chromosome even smaller than that of strain 22 resulted in seemingly unstable cells (Figure 2B, strain 20). Comprehensive iteration analyses combined with fluorescent microscopy studies to identify intermediates in chromosome replication, segregation, and segmentation should help to address the uncertainties above. Although not addressed in this work, it would be useful to investigate whether the *telN* gene could be integrated into one or multiple chromosomes, thereby avoiding the use of additional episomes. As an added advantage, linear, but not circular, chromosomes, require a single plasmid-encoded protein, TraB, to be transferred via conjugation.[19]

An interesting challenge would be to eliminate the slight growth defect of the strain with two chromosomes. Strain 22 + *telN* has a chromosome distribution similar to that of *V. cholerae*, which has a doubling time that can be as short as 18 min.[20] A few differences, though, distinguish the chromosome configuration of these two organisms. Perhaps the most important one is that whereas *V. choloerae*'s chromosomes are circular, strain 22 + *telN* has linear chromosomes. This can affect the doubling time in various ways. For example, we have seen that whereas OriV can sustain the replication of a circular 4.6 Mbp chromosome, it is unable to replicate an otherwise identical linear chromosome (Figure 2B, strain 16). Accordingly, an interesting hypothesis to test is whether normal cell growth is restored by circularizing the chromosome with the heterologous replication origin. Such a configuration would still be compatible with our proposal of chromosome fragmentation. In addition, a circular chromosome would enable the biological function of a bidirectional terminus region.[21] Finally, finding out the ratio of the two chromosomes' copy number would help to determine the rate-limiting step in the doubling time of the novel strain.

We envision that diverse bacterial subgenome fragments with sizes of up to 1 to 2 Mbp could be mixed and introduced into compatible chassis using yeast as the DNA assembly (donor) organism and a combination of selectable and counterselectable markers. Additional origins of replications with DNA capacities of over 1 Mbp, potentially compatible with those described in this work, might be utilized. Examples include those present in those α and β proteobacteria harboring multiple chromosomes such as *Agrobacterium tumefaciens*, *Sinorhizobium meliloti*, *Rhodobacter sphaeroides*, and *Burkholderia cepacia* among others.[12] Bacterial artificial chromosomes based on the F′ episome, although compatible with this approach, are restricted to fragment sizes significantly smaller than 1 Mbp because of their limited DNA capacity.[22]

A fundamental need of the synthetic biology field is a genome-engineering toolbox with its corresponding reagents that allows the manipulation of chromosomes with the same simplicity and confidence as that with which we modify plasmids today. The fragmentation of the *E. coli* genome into smaller viable units harboring essential functions brings the above ambition one step closer to reality.

METHODS

Strain Engineering. *E. coli* strain MG1655[23] was used as a starting point for the construction of all of the engineered strains. Exogenous DNA elements were introduced via lambda red recombineering.[24] Briefly, the corresponding DNA fragments were linked to a kanamycin selection marker and precloned into pUC19, from where the final DNA fragments using for recombineering were PCR-amplified. The *E. coli* origin of replication was PCR-amplified from strain MG1655 (nucleotides 3 923 768−3 923 998 of GenBank accession no. U00096). The N15 bacteriophage *tos* element was prepared by de novo gene synthesis on the basis of the the published sequence (nucleotides 24 474−25 038 of GenBank accession no. AF064539). The *V. cholerae* replication origin of chromosome II was PCR-amplified from genomic DNA of strain O1 biovar El Tor N16961 (ATCC, Manassas, VA,[13] nucleotides 1 069 875−3190 of GenBank accession no. AE003853). For sequences and additional details on strain design and construction, see the Supporting Information. The positions of the elements in the MG1655 are shown in Table 1.

Table 1. Position of the DNA Elements in the MG1655 Chromosome

DNA element	position[a]
tos1	1 585 783
tos4	296 421
tos5	651 211
tos7	2 956 901
OriC1	959 401
OriC2	4 422 941
OriC3	343 291
OriC4	1 989 748
OriV1	959 401
OriV2	4 422 941
OriV3	343 291
OriV4	2 507 555

[a]Numbers refer to the position in the published sequence (GenBank accession no. U00096).

Southern Blot. Genomic DNA was isolated from the engineered strains using the PureLink Genomic DNA kit (Life Technologies, Carlsbad, CA), digested with *Hin*dIII, and run on 1% agarose gels. Transfer onto BrightStar-Plus Membranes (Life Technologies) was performed using the iBlot Dry Blotting System (Life Technologies) following the manufacturer's directions. Probes were PCR-amplified using primers described in the Supporting Information and labeled using the BioPrime DNA Labeling System (Life Technologies). Membranes were processed, and DNA was visualized using the BrightStar BioDetect Kit (Life Technologies). The biotinylated 2-Log DNA Ladder (0.1−10 kb) was from New England Biolabs (Ipswich, MA).

Pulse-Field Gel Electrophoresis. Agarose-embedded genomic DNA was prepared using the CHEF Bacterial Genomic DNA Plug Kit (Bio-Rad, Hercules, CA) and transferred to the wells of a 0.8% TAE agarose gel. Electrophoresis was performed using a CHEF-DR II System with a cooling module (Bio-Rad, Hercules, CA) with initial and final switching times of 250 and 900 s, respectively. The voltage was set at 3 V/cm. Following the 50 h run, the gel was stained with ethidium bromide and visualized by standard imaging techniques. CHEF DNA size marker *H. wingei* (Bio-Rad) was used as a DNA standard (1−3.1 Mb).

Grow Curves and Microscopy. Overnight cultures of the strains were inoculated into fresh, prewarmed LB broth at an initial OD_{600} of 0.01 and incubated at 37 °C with shaking (200 rpm). Their optical densities at 600 nm were measured at 2 h intervals. Experiments were performed in duplicate. Grow rates

(k) were calculated by applying the formula $k = \ln N_1 - \ln N_0/(t_1 - t_0)$, where N_0 and N_1 represent the culture's OD_{600} at t_0 and t_1 after inoculation. For MG1655, MG1655 + *telN*, and strain 22, t_0 and t_1 were 2 and 4 h, whereas for strain 22 + *telN*, t_0 and t_1 were 4 and 6 h, respectively. Fifty microliters of an overnight culture was concentrated to 10 μL by centrifugation followed by spreading on a glass slide. After air-drying, 50 μL of HistoMount mounting solution (Life Technologies) was applied to the sample, and the sample was covered by a coverslip. The cell morphology was visualized with a 100× oil objective under a bright-field NIKON Eclips E400 microscope.

Other Reagents. Oligonucleotides (see Supporting Information) were from Life Technologies. PCR amplification assays were conducted using AccuPrime Pfx DNA polymerase (Life Technologies). The plasmid pJAZZ-OC was from Lucigen (Middleton, WI).

■ ASSOCIATED CONTENT

Supporting Information

Strain engineering methodologies; DNA fragments for gene replacement and southern blot probes; element sequences; oligonucleotide sequences; oligonucleotides for validation of chromosome fragmentation across tos elements; and DNA sequences of the elements used in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): The authors hold financial interests in Life Technologies.

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